[3] *Division of Biosignaling*
[4] *Division of Medicinal Safety Science*
National Institute of Health Sciences
Tokyo, Japan

[5] *Division of Genomic Medicine*
Department of Advanced Biomedical Engineering and Science
Tokyo Women's Medical University
Tokyo, Japan

*Address correspondence to this author at: Division of Biochemistry and Immunochemistry, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Fax 81-3-5717-3832; e-mail yoshiro@nihs.go.jp.

DOI: 10.1373/clinchem.2006.077990

Preanalytical Stability of Adrenocorticotropic Hormone Depends on Time to Centrifugation Rather than Temperature

To the Editor:

Preanalytical factors can affect reliability of hormone assay results. Adrenocorticotropic hormone (ACTH) in blood is considered highly unstable because of proteolytic degradation *(1–4)*, so storage of blood samples on ice until analysis is recommended. In clinical practice, however, this procedure may present logistical problems because most samples for ACTH measurement must be shipped from the place of sample collection to the laboratory. Therefore, we studied the impact of time and temperature before plasma separation and analysis on the results of ACTH assays.

At 8 AM, we obtained 2 blood samples from each of 19 healthy volunteers and 2 patients with pathologically high ACTH values (1 with Addison disease and 1 with congenital adrenal hyperplasia). Volunteers and patients gave written informed consent, and the ethics committee for our institution approved the study. ACTH concentrations were 5–774 ng/L. As recommended by the manufacturer (Monovette, Sarstedt), collection tubes contained 1.2–2 g of potassium EDTA/L, with a maximum 1% dilution effect of liquid EDTA.

For each set of 2 samples, 1 sample was centrifuged immediately after collection and then divided into aliquots for storage at room temperature (22 °C), 4 °C, or −20 °C for 1, 2, 4, 24, or 48 h before being frozen at −80 °C until it was assayed. The 2nd sample was left in the primary collection tube at either room temperature or 4 °C for 1, 2, 4, 24, or 48 h before centrifugation and then frozen at −80 °C until it was assayed. All samples from 1 individual were analyzed in 1 run with an automated chemiluminescence assay (Advantage, Nichols). Results were compared with the concentration obtained from an aliquot stored under standard conditions (collected on ice, immediately centrifuged, and frozen at −80 °C until analysis) and expressed as percentage of standard condition.

We used pairwise 1-sided testing with the Wilcoxon signed-rank test to analyze the significance of changes in hormone concentrations. The duration of hormone stability was approximated by fitting a monoexponentially decaying function to the raw data for each scenario and calculating the time period of 10% decreases in hormone concentrations compared with baseline concentrations under standard conditions. Analytical testing and curve fitting were implemented in Mathematica version 5 (Wolfram Research).

As expected, measured ACTH concentrations significantly decreased with time before freezing at −80 °C. Interestingly, temperature alone did not appear to influence hormone concentration stability ($P > 0.05$). The calculated times for decay of mean concentrations to 90% of baseline values at 4 °C and room temperature, respectively, were 24 h and 19 h for uncentrifuged samples and 33 h and 31 h for immediately centrifuged samples.

After 2 h of storage at 4 °C, the ACTH concentration was significantly higher in samples centrifuged immediately than in uncentrifuged samples ($P < 0.01$) (Fig. 1A). At 22 °C this difference was observed after 1 h ($P < 0.05$) (Fig. 1B). The decrease in the measured ACTH concentration with time before centrifugation was also observed in samples containing

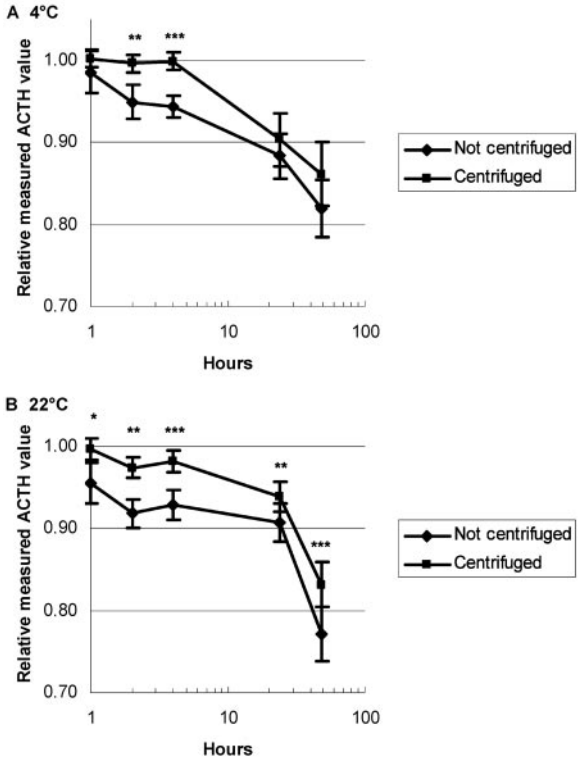

Fig. 1. ACTH concentrations drop significantly faster if samples are not centrifuged after venipuncture [mean (SE)]; temperature per se is less important.

very high concentrations of ACTH. There was no difference in mean decay time in samples of controls and patients.

We found a significant change in ACTH plasma concentrations with time, as in other studies *(1, 5)*, but this change was much smaller than expected. Unlike another investigation *(5)*, we studied samples not only from healthy volunteers but also from patients with high ACTH concentrations. Our study did not show a difference in the mean rate of hormone concentration change in high-ACTH samples vs normal samples. In our study, for up to 24 h the decline in the measured ACTH concentration was ≤10% even in whole blood stored at room temperature. Given the analytical imprecision of ≤15%, commonly accepted for immunoassays, a 10% change in the hormone concentration attributable to preanalytical factors seems not to be a major problem in a clinical setting. We therefore confirm stability of ACTH in EDTA plasma for ≤24 h as previously reported *(5)* for a manual radioactive version from the same assay from the same manufacturer. Sample temperature during the preanalytical phase appears to have less influence on measured ACTH concentrations than does time to centrifugation. We speculate that enzymes involved in EDTA degradation are not inhibited sufficiently at 4 °C.

Although the mean decay in measured ACTH concentration after storage for 24 h at room temperature without centrifugation was only 10% [mean (SD), 9% (11%)], the decrease was >20% in samples from 3 healthy volunteers and was not prevented by storage at 4 °C. No relevant change occurred in any of the samples during the first 4 h, however. For clinical practice we therefore recommend that centrifugation and separation of plasma supernatant be performed within 4 h of sample collection. Cooling of samples seems to be much less effective. Thus, the preanalytical procedure can be simplified without risking clinically relevant changes in measured hormone concentrations.

We acknowledge Brigitte Mauracher, Rita Schwaiger, Sarina Meurer, and Juliane Ramisch for excellent technical assistance.

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Nicole Reisch
Martin Reincke
Martin Bidlingmaier*

Department of Endocrinology
Medizinische Klinik Innenstadt
University of Munich
Munich, Germany

*Address correspondence to this author at: Department of Endocrinology, Medizinische Klinik Innenstadt, University of Munich, Ziemssenstrasse 1, D-80336 Munich, Germany. E-mail martin.bidlingmaier@med.uni-muenchen.de.

DOI: 10.1373/clinchem.2006.080622

Indican Interference in Bilirubin Assays: A Classical Solution Still Applies

To the Editor:

Abbott Laboratories recently supplied a new reagent for total bilirubin (catalog no. 6L45-20) for use with the Architect *c*Systems analyzer. The reagent uses 2,4-dichlorophenyl diazonium (2,4-DCPD) and is described as minimizing interference from hemoglobin, although interference from indican was reported to be higher than with the previous reagent *(1)*. Our initial comparison of the new and previous reagents yielded a regression equation with a slope of 1.05 (Fig. 1, upper panel, ■) and similar imprecision (not shown) for the 2 reagents. After introduction of the new reagent into routine use, however, bilirubin results in renal dialysis patients were noted to be higher than with the previous reagent. Among ~512 predialysis samples received during a 3-day period from patients on renal dialysis, 43% had bilirubin values above the upper limit of the reference interval (3–13 mg/L). For most of these patients, bilirubin concentrations reported within the previous 1–2 months had been within the reference interval.

We measured bilirubin with both the old and new reagents in a group of predialysis renal patient plasma specimens (Fig. 1, upper panel, □). The slope of the Deming regression equation was significantly higher in the renal group than it was in the initial method-comparison study using unselected leftover laboratory specimens (1.33 vs 1.05; unpaired *t*-test, $P < 0.0001$). Interestingly, with the new reagent the absorbance continued to increase after the first minute in renal dialysis samples, but not in nonrenal samples (Fig. 1, middle panel).

The findings suggested interference from indican, a metabolite that increases in uremia *(2)*. After addition of indican, total bilirubin results with the 2,4-DCPD and 2,5-DCPD methods were reported to increase by 50 and 33 mg/L per mmol/L of indican, respectively *(2)*. Abbott reported *(1)* that, with the new reagent, the bilirubin increased by 15 mg/L for 0.25 mmol/L of added indican as compared to a 17 mg/L increase for 0.50 mmol/L of indican using the old reagent. Indican concentrations up to 0.38 mmol/L have been observed in predialysis serum samples from renal failure patients *(3)*.

To test the effect of indican, we added indican (indoxyl sulfate; Sigma-Aldrich) to a plasma pool generated from patients with normal renal function. The time course of absorbance for the new Abbott bilirubin assay matches that seen during analysis of plasma from dialysis patients. In the absence of added indican,